COMPLEMENT DEVIATION IN ROCKY MOUNTAIN SPOTTED FEVER.*

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The experiments here described have been made at various times in this laboratory, the original object being to provide a basis for an early diagnostic test for Rocky Mountain spotted fever.

The first experiments were carried out by Liborio Gomez at the suggestion of Dr. Ricketts and we shall give a brief outline of his unpublished results. Gomez used an ox-rabbit system, using the serum from infected guinea-pigs as antigen (drawn during second to fourth day of fever) and immune serum from guinea-pigs and rabbits as antibody. He first determined that the serum containing the antigen and the serum containing the antibody possessed no hemolytic amboceptors for ox corpuscles. His experiments then followed the method of Bordet-Gengou. A positive deviation was obtained in the case of one guinea-pig which had recovered from the disease and which had received an immunity test some ten weeks before being killed. In a recovered rabbit he also obtained a positive though weak fixation of the complement. In two guinea-pigs killed four or five months after recovery from a single attack of spotted fever Gomez found a retardation of hemolysis of from two to five hours, as compared with controls of immune serum and virus alone.

In his experiments with the immune guinea-pig serum Gomez left the tubes containing the complement-antibody-antigen mixture in the incubator for over 14 hours before adding the hemolytic system. This period of time is long and leaves some doubt as to the value of the result obtained. Gomez notes that the smaller doses of antigen-antibody recommended by Bordet and Gengou failed to give results. In his experiments with the blood of a recovered rabbit he established a binding with 0.6 c.c. of the serum. Gomez notes, however, that large doses of antibody (1–2 c.c.) will,

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when mixed with complement, bind or destroy its action without the presence of antigen.

With the idea of further testing this reaction, Dr. Ricketts and Mr. R. M. Wilder made several experiments, using the eggs of infected ticks as antigen. It has already been shown that the eggs of infected ticks are rich in an organism which is supposedly the cause of Rocky Mountain spotted fever and it was supposed that these eggs would therefore afford a suitable source of antigen. In these experiments a goat-rabbit hemolytic serum was used and the exact dosage of fresh, normal guinea-pig complement was fixed for each experiment.

In the first three experiments of the series eggs were used in quantities varying from 1 to 100, the antibody being varied from 0.05 to 0.1 c.c. As an example of these experiments we insert Table 1.

From these experiments it will be noted in Group A (Table 1) that there is a slight decrease in hemolysis as the number of eggs is increased. In Group B with a larger amount of antibody the same result is noted, although the total amount of hemolysis is greater in each corresponding tube than in Group A. The degree of hemolysis is not dependent on the number of eggs, since Group C shows a constant amount of hemolysis in each tube. Evidently there was some reactivation[1] of the heated serum by the unheated, and this reactivation was greater when the amount of heated complement in the antibody (guinea-pig serum) was greater. This was borne out by the results in tubes 17 and 18 in which a large amount of antibody increased the hemolytic action of 0.01 c.c. of complement. In this experiment the total amount of complement used was not quite sufficient to cause complete lysis, as shown in tube 13.

In the second experiment of Ricketts' series larger numbers of eggs were used (75–100); the results were again inconclusive. It was found that the eggs alone, without the addition of antibody, were capable of preventing lysis. It was again noted that antibody and complement, when together, were more hemolytic than com-

[1] That such an activation takes place was shown in an experiment in which we first established the minimum dosage of normal complement and then added varying amounts of antibody to a subminimal dose of complement. Doses of inactivated antibody from 0.01 c.c. up caused complete laking in the tubes, although alone the antibody had absolutely no effect.

plement alone, and it was assumed that the antibody contained hemolytic amboceptors which were activated by the complement. An effort was made in the third experiment to absorb the hemolytic amboceptor supposed to be present in the antibody, but the results

TABLE 1.

COMPLEMENT DEVIATION IN SPOTTED FEVER.

Groups	Sensitized Corpuscles	Antibody	Antigen (Eggs)	Complement	Results
Group A					
1.....	0.5	0.05	1	0.01	Marked hemolysis (75 per cent)
2.....	0.5	0.05	10	0.01	Marked hemolysis (less than tube 1)
3.....	0.5	0.05	20	0.01	Marked hemolysis (less than tube 1)
4.....	0.5	0.05	50	0.01	Moderate (less than 2 or 3)
Group B					
5.....	0.5	0.1	1	0.01	Complete
6.....	0.5	0.1	10	0.01	Complete
7.....	0.5	0.1	20	0.01	Complete
8.....	0.5	0.1	50	0.01	Almost complete
Group C					
9.....	0.5	0	1	0.01	Moderate
10.....	0.5	0	10	0.01	Moderate
11.....	0.5	0	20	0.01	Moderate
12.....	0.5	0	50	0.01	Moderate
Group D					
13.....	0.5	0	0	0.01	Marked hemolysis (75 per cent)
14.....	0.5	0	0	0	0
Group E					
15.....	0.5	0.05	0	0	0
16.....	0.5	0.1	0	0	0
Group F					
17.....	0.5	0.05	0	0.01	Almost complete
18.....	0.5	0.1	0	0.01	Complete
Group G					
19.....	0	0	0	0.01	0 (solution clear)
20.....	Unsensitized	0	0	0.01	0

The following materials were used: Sensitized erythrocytes from goat, washed, in 5 per cent suspension, 1 day old; complement—fresh, normal guinea-pig serum; antibody—inactivated serum of guinea-pig 2,537, recently recovered from spotted fever; antigen—eggs of *D. Modestus* (56 [57], 11, F. 4), 10 days old, rich in bacilli.

The goat corpuscles were sensitized by adding 0.8 c.c. of immune rabbit serum to 20 c.c. of a 5 per cent suspension, incubating 20 minutes and washing once. This was done in order to avoid the necessity of making separate measurements of corpuscles and of the hemolytic serum.

Antigen+antibody+complement was allowed to incubate 20 minutes (1 hr. in Experiment 3) in a total volume of 0.3 c.c.; 5 c.c. suspension of corpuscles was then added and the total volume raised to 2 c.c. by the addition of normal salt solution. The mixture thus prepared was incubated 2 hours and then placed on ice over night.

were negative. In a fourth experiment dried serum of an immune horse was dissolved and used as an antibody, giving a fairly strong deviation with 0.2 c.c., but it was found on further tests that 0.4 c.c. of this serum alone would bind all the complement. This was possibly due to the presence of an anti-complement in the serum of

the horse, it having been actively immunized by the injection of virulent guinea-pig blood.

We have repeated these experiments with tick eggs, crushing a given number of them (100) in sterile salt solution and then using decreasing dilutions of this emulsion. Three series of eggs were used: (1) eggs from infected ticks (showing large numbers of organisms); (2) eggs from normal ticks; and (3) eggs from ticks raised on immune pigs. The three series, however, showed complete lysis. It is true that no lysis was obtained in the 100 per cent emulsion, but it was found that tubes without antibodies would give the same result. In these experiments the antigen-antibody-complement mixture was permitted to incubate as long as 3–3½ hours.

All the results so far obtained, while showing some deviation, were not as clear-cut as might be expected in a disease like spotted fever in which the serum of recovered animals possesses such a marked protective power.[1] We therefore undertook the testing of various tissue extracts and of sera taken from animals during fever, in the hope of finding an efficient antigen. The inactivated serum of recovered guinea-pigs served as antibody. An ox-rabbit hemolytic system was used, the corpuscles being first sensitized, and the exact dosage of complement established for each experiment. In the case of various bacterial suspensions used as antigens, a standardized loop of a 24-hour agar culture was always made up in 1 c.c. of normal salt solution.

In one experiment varying amounts of a watery extract of macerated lymph glands, spleen, and testicle of a guinea-pig which had died of spotted fever were used as antigen, with varying amounts of antibody (0.025 to 0.1 c.c.), but there was no deviation.[2] This negative result led us to investigate whether the antibody was thermostable or whether it had been destroyed by the heating at 56° C. We found that both fresh and heated immune sera protected equally well. Evidently the difficulty remained in the antigens. We therefore made separate extracts

[1] Ricketts and Gomez, *Jour. Infect. Dis.*, 1908, 5, pp. 221–44.

[2] It may be stated here that the most marked internal changes during a course of spotted fever in the guinea-pig occur in the lymph glands, testicle, and spleen. Large numbers of bodies similar in appearance to the bacillus found in the tick eggs can be seen in the spleen, lymph glands, and liver when sections are stained with Giemsa (Dr. Maria B. Maver). The organism must also be present, of course, in the blood, which is highly virulent, but it has not been seen equally well here in stained preparations.

from the macerated material of infected pigs, using serum, lungs, liver, spleen and lymph glands, kidneys, muscles, brain, and intestine. Varying quantities were used (0.1 c.c. to 0.5 c.c.), with a constant amount of immune guinea-pig serum. The organ extracts were not hemolytic. The results of the experiment were again negative. In connection with these experiments a series was made with a suspension of the culture of the "Spaniard" organism[1] as antigen and the serum of guinea-pigs recovered from infection with it and from Rocky Mountain spotted fever as antibody.

As will be seen from Table 2 complete binding of the complement was obtained with both antibodies. While this was to be ex-

[1] The "Spaniard" organism, culturally, morphologically, and immunologically, seems to be practically identical with a stock culture of *B. cholera suis* obtained from the Department of Bacteriology of the University of Chicago. It crept into the spotted fever passage virus derived from the patient "Spaniard" and gradually replaced the spotted fever virus. Whether it actually outgrew and "smothered" the virus is not known. The "Spaniard" patient was suffering from the Idaho spotted fever—a strain which on three previous occasions it had been impossible to keep going by passage through the guinea-pig—so that the adventitious infection may not have been responsible for the "dying out" of the spotted fever virus. By proper attention to dosage, the infection produced in the guinea-pig by the intraperitoneal injection of the "Spaniard" organism can be made to parallel very closely in some respects a case of spotted fever. There is the same three days' incubation period, the subsequent high fever, and death in about 10 days. Post mortem the enlarged, hemorrhagic lymph glands and swollen spleen are conspicuous. The gangrenous ears and scrotum, the cutaneous eruptions, and the hemorrhages in the region of the pampiniform plexus so common in spotted fever are wanting, while a fibrinous exudate in the peritoneal cavity, never found in the latter disease, is always present. In non-fatal cases the temperature of spotted fever pigs returns to normal in about 10 days and there remains; in non-fatal infections with the "Spaniard" organism, a low fever may persist for 20 days to a month with occasional exacerbations. Cultures from all spotted fever cases remain sterile; the specific bacilli may always be cultivated from the heart's blood, peritoneal exudate, and spleen of pigs dying from infection with the "Spaniard" organism. Finally, recovery from spotted fever does not confer immunity to the "Spaniard" organism, though a high grade of specific immunity is produced. Both cause a "hemorrhagic septicemia" but the "Spaniard" organism is much less prone to produce hemorrhages than is the spotted fever virus.

It is a striking coincidence that in three diseases produced by micro-organisms which apparently cannot be cultivated, namely yellow fever, Rocky Mountain spotted fever, and hog cholera, and the first two of which at least may be transmitted by insects, cholera-suis-like organisms should have intruded themselves. This fact, coupled with those detailed in this paper on the comparative fixation tests, suggests that there may be something more concerned here than mere coincidence. There may be an actual relationship of some kind between the various causal organisms. With this idea in mind the following preliminary experiment with precipitins is of interest:

The precipitogens consisted of filtered, 24-hour broth cultures of *B. cholera suis*, "Spaniard," *Staph. aureus*, and *B. typhosus:* the precipitins were contained in the unheated sera of spotted fever immune guinea-pigs Nos. 2,658 and 2,381 and of "Spaniard" immune guinea-pigs Nos. 2,342 and 2,669. Precipitates formed in those tubes containing the cholera suis and "Spaniard" filtrates with the spotted fever and "Spaniard" immune sera alike in a serum dilution of 1–20 and not at all in the staphylococcus and typhoid tubes. Control tubes remained clear.

SAMPLE PROTOCOLS.

Precipitogen	Precipitin	NaCl Sol.
0.5 c.c.	0.01 c.c.	1.49 c.c.
0.5 c.c.	0.1 c.c.	1.4 c.c.
0.5 c.c.	0	1.5 c.c.
0	0.1 c.c.	1.9 c.c.

pected in the "Spaniard-Spaniard" combination, the "Spaniard"-spotted fever result was rather unexpected. While these controls had been intended principally as a check on our technic by the addition of known factors, and showed that our method was correct, the result indicated either that the "Spaniard" culture was a specific antigen for Rocky Mountain spotted fever, or stood in a group relationship to the same (we knew that the first supposition was not true) or that the guinea-pigs had at some previous time been infected with the "Spaniard" bacillus and were therefore immune.

TABLE 2.

COMPLEMENT DEVIATION WITH "SPANIARD" BACILLUS AND SPOTTED FEVER AND "SPANIARD" IMMUNE SERUM.

SENSITIZED CORPUSCLES	ANTIBODIES		ANTIGEN CULTURE "SPAN." VI	COMPLEMENT	RESULTS
	Sp. Fever 2,517	"Spaniard" 2,343			
0.5	.....	0.025	0.05	0.01	No hemolysis
0.5	.....	0.025	0.1	0.01	No hemolysis
0.5	.....	0.025	0.25	0.01	No hemolysis
0.5	.....	0.025	0.5	0.01	No hemolysis
0.5	.....	0.05	0.25	0.01	No hemolysis
0.5	.....	0.1	0.25	0.01	No hemolysis
0.5	.....	0.0	0.5	0.01	Complete
0.5	.....	0.1	0	0.01	Complete
0.5	0.025	.....	0.05	0.01	No hemolysis
0.5	0.025	.....	0.1	0.01	No hemolysis
0.5	0.025	.....	0.25	0.01	No hemolysis
0.5	0.025	.....	0.5	0.01	No hemolysis
0.5	0.05	.....	0.25	0.01	No hemolysis
0.5	0.1	.....	0.25	0.01	No hemolysis

Tubes incubated for 30" and corpuscles then added and tubes made up to 2 c.c. with normal salt solution.

Incubated 1 hr. 30" and then placed at room temperature over night.

This experiment was repeated using the same antibodies and fresh suspensions of "Spaniard," typhoid bacilli, and *Staphylococcus aureus* as antigens. The results with the typhoid bacilli and staphylococci were absolutely negative but there was deviation as in the previous experiment with the "Spaniard" organism.

The effect of large doses of antibody causing an increase of hemolysis, as noted in the Ricketts experiments already quoted, was observed.

Certain preliminary experiments indicated that the bacillus of hog cholera, when used as an antigen with Rocky Mountain spotted fever and "Spaniard" immune sera as the antibodies, would give

evidence of binding the complement. A quantitative experiment was performed in order to compare this binding with the results obtained using the "Spaniard" culture as antigen. These results are shown in Table 3. As will be seen in Series A (spotted fever-"Spaniard"), there is first a progressive decrease in the amount of

TABLE 3.

GROUPS	EXPERIMENT 12 SENSITIZED CORPUSCLES	ANTIBODIES		ANTIGENS		COMPLEMENT	RESULTS
		Sp. Fever 2,381	"Spaniard" 2,342	Span. VI	B. cholera suis		
Group A							
1.....	0.5	0.005	.....	.05	....	0.02	Incomplete laking
2.....	0.5	0.0075	.....	.05	....	0.02	Incomplete laking
3.....	0.5	0.01	.....	.05	....	0.02	Trace of laking
4.....	0.5	0.02	.....	.05	....	0.02	Trace of laking
5.....	0.5	0.03	.....	.05	....	0.02	Trace of laking
6.....	0.5	0.04	.....	.05	....	0.02	0
7.....	0.5	0.05	.....	.05	....	0.02	Slight laking
8.....	0.5	0.1	.....	.05	....	0.02	Moderate laking
Group B							
9.....	0.5	0.005	.....	...	0.05	0.02	Incomplete laking
10.....	0.5	0.0075	.....	...	0.05	0.02	Incomplete laking
11.....	0.5	0.01	.....	...	0.05	0.02	Incomplete laking
12.....	0.5	0.02	.....	...	0.05	0.02	Trace of laking
13.....	0.5	0.03	.....	...	0.05	0.02	Trace of laking
14.....	0.5	0.04	.....	...	0.05	0.02	Least laking
15.....	0.5	0.05	.....	...	0.05	0.02	Slightly more laking
16.....	0.5	0.1	.....	...	0.05	0.02	Slightly more laking
Group C							
17.....	0.5	0	0.005	.05	....	0.02	Incomplete laking
18.....	0.5	.....	0.0075	.05	....	0.02	Slight laking
19.....	0.5	.....	0.01	.05	....	0.02	Slight laking
20.....	0.5	.....	0.02	.05	....	0.02	0
21.....	0.5	.....	0.03	.05	....	0.02	Complete laking
22.....	0.5	.....	0.04	.05	....	0.02	Complete laking
23.....	0.5	.....	0.05	.05	....	0.02	Complete laking
24.....	0.5	.....	0.1	.05	....	0.02	Complete laking
Group D							
25.....	0.5	.....	0.005	...	0.05	0.02	Complete laking
26.....	0.5	.....	0.0075	...	0.05	0.02	Complete laking
27.....	0.5	.....	0.01	...	0.05	0.02	Complete laking
28.....	0.5	.....	0.02	...	0.05	0.02	Complete laking
29.....	0.5	.....	0.03	...	0.05	0.02	Complete laking
30.....	0.5	.....	0.04	...	0.05	0.02	Complete laking
31.....	0.5	.....	0.05	...	0.05	0.02	Complete laking
32.....	0.5	.....	0.1	...	0.05	0.02	Complete laking

laking reaching a maximum in tube 6 in which we find complete binding and there follows increased laking in tubes 7 and 8. Series B (spotted fever+hog cholera) shows a similar reaction, though the best tube still shows a trace of laking. In Series C ("Spaniard"+ "Spaniard") we find a marked deviation in the first four tubes (90 per cent to 100 per cent) and complete laking in the last four tubes. Series D, on the other hand ("Spaniard"-hog cholera), gave complete laking. This experiment was repeated twice, using sera from different immune pigs, and similar results were obtained with the

exception that now the "Spaniard"-hog cholera combination gave the reverse of the result in Experiment 4, in which we found a negative reaction. Further experiments have confirmed these results, and also seem to show (1) that the serum of normal guinea-pigs does not contain the specific antibody; (2) that the spotted fever immune serum causes no deviation in the presence of typhoid, anthrax, dysentery (Shiga), and Friedländer bacilli, staphylococci, and cholera germs, although it has a specific affinity for certain strains of bacteria of the hog cholera group. The spotted fever antibody, in certain proportions, will bind complement with the hog cholera and "Spaniard" bacilli, and the "Spaniard" immune serum will bind with the same bacilli. We determined, however, that pigs immune to Rocky Mountain spotted fever are not protected against the "Spaniard" organism.

We have failed to find a parallelism between the bacteriolytic power of these immune sera on hog cholera bacilli and the "Spaniard" bacillus, as determined by the Neisser-Wechsberg technic, and their power to deviate complement. That is, the bactericidal power of normal sera and of "Spaniard" immune sera was almost absolutely identical, while only the immune sera supplied the amboceptor necessary for the deviation of the complement. Apparently the presence of substances in sera which cause a fixation or deviation of complement need not imply the presence of bacteriolytic or protective antibodies. A somewhat similar phenomenon has been observed by Torrey and others.[1]

CONCLUSIONS.

Positive deviation of complement, using spotted fever antigen and antibody, has been obtained in but one instance, and the results of experiments undertaken to confirm this positive finding have been negative.

The negative results are probably to be explained by the small quantities of organisms present in any one preparation of antigen and by the admixture of various substances in the antigens which in themselves absorb or neutralize complement, this in turn pro-

[1] Torrey, *Jour. Med. Res.*, 1910, 22, p. 95.

hibiting the use of the large doses of antigen which might otherwise be effective.

The protective power of spotted fever immune serum is not destroyed by heating to 56° C. for 30 minutes.

An apparent reactivation of heated serum (antibody) was met with in many cases which, by causing hemolysis, tended to mask results. This might become a source of error in work of this kind.

An organism probably identical with the hog cholera bacillus replaced the spotted fever virus in one of the passages. The serum of guinea-pigs immune to spotted fever and of guinea-pigs immune to this organism deviated the complement alike when a suspension of the above bacterium was used as antigen. This fact, together with other considerations mentioned in this paper, suggests that there may be a definite relationship of some kind between the virus of Rocky Mountain spotted fever and the hog cholera bacillus.

Our results suggest, further, that the presence of substances in sera which cause a complement-fixation need not imply the presence of bactericidal or protective antibodies.